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Glutathione-s-transferase (GST)- π gene is frequently methylated in prostate cancer and recent evidence has linked aberrant expression of this gene with the development of prostatic adenocarcinomas (PACs). The main purpose of this study is to evaluate GST- π protein expression by immunohistochemistry (IHC) and develop a simple DNA amplification assay for detection of GST- π methylation status. 220 cases including 180 PACs and 20 cases each of normal and benign hyperplastic tissues were identified. Protein expression was evaluated on 4 μ m paraffin sections in all cases using a polyclonal rabbit antihuman GST- π antibody by an automated method. Methylation status was assessed in 24 cases including 18 PACs and 6 benign tissues using the 466/468 primer pair by a gel based PCR amplification method. 175 of 180 (97%) PACs demonstrated loss of GST- π protein expression in comparison to the presence of this protein in 100% of benign tissues including normal and BPH specimens. 7 of 18 (39%) cases of PACs and 1 of 6 (17%) benign tissues demonstrated methylated GST- π promoter DNA. Given that the overwhelming majority of PACs lacked GST- π protein expression, no correlation was found with any prognostic variables. In conclusion, GST- π protein is downregulated in almost all prostate cancers and is associated with methylation of the promoter region of the gene. Further studies are warranted to evaluate the utility of GST- π methylation assay in screening and diagnosis of prostate cancer.

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Introduction

Prostate cancer is currently the most commonly diagnosed cancer in American men. Early diagnosis through accurate screening techniques will significantly contribute to the successful management and eventual eradication of this disease. Currently, serum PSA determination remains the cornerstone of prostate cancer screening. However, more accurate screening to better differentiate patients with benign disease from those with prostatic cancer will alleviate the unnecessary surgical procedures with a significant impact on patient morbidity and health care costs. Glutathione-S-transferases (GSTs) are a family of detoxification enzymes which catalyze the conjugation of a wide variety of endogenous and exogenous toxins with glutathione. Recent literature reported that methylation of the GST- π gene, which inactivates the expression of the enzyme, is a cancer specific marker in the prostate (1). Since the original submission of the current research project, considerable new evidence has been published linking aberrant expression of the GST- π gene with the development of prostate cancer (2, 3). In a recent study of prostate cancer tissues using a PCR based assay for GST- π methylation, 24/32 (75%) cases were positive and the results correlated with disease stage (4). It has also now been shown that GST- π methylation is not confined to specific sites in the promoter, but occurs throughout the entire CpG island sequence (5). Interestingly, hypermethylation is regularly identified in the GST- π gene in normal and hyperplastic prostate tissues outside the promoter region with no loss of gene function (5). The main purpose of this study is to evaluate GST- π protein expression by immunohistochemistry and develop a simple quantitative DNA

amplification assay for detection of GST- π methylation status. The scope of this study is to newly develop both an automated immunohistochemistry system and a quantitative PCR based assay and apply these assays to prostate cancer tissues which constitute part of the established data base at the Albany Medical College. The ultimate goal will be to detect circulating methylated GST- π DNA bearing prostate cancer cells. When accomplished, this assay will be tested for its ability to serve as a sensitive and specific screening test for prostate cancer. Additionally, application of this assay to tiny pre-operative needle biopsy specimens will significantly aid in the definitive diagnosis of prostatic carcinoma.

Body

Task 2. Assemble and blind slides and microdissected tissue samples from ≥ 180 prostate tumor tissues in the previously characterized Albany Medical College (AMC) collection, as well as ≥ 20 normal and \geq BPH prostate tissue samples.

Task accomplished

180 prostatic adenocarcinomas and 20 cases each of normal and benign prostatic hyperplastic tissues were identified from the established database at the Albany Medical College. H&E slides in each case were reviewed to confirm the diagnosis. The tumors were graded (Gleason grade) and staged (AJCC). Tumors were classified as high grade when the Gleason score was 7 and above and as low grade when the score was 6 and below. Serum PSA levels and clinical follow-up were obtained from the patients' records. A post-surgical elevation of PSA level from a baseline of 0 ng/ml to >0.4 ng/ml was considered as disease recurrence. Tissue from 20 patients who underwent transurethral resection for a clinical diagnosis of benign prostatic hyperplasia were included. The 20 cases of unremarkable prostatic tissue were acquired from autopsy material on patients who died of unrelated causes. Approximately 200 μ m of paraffin-embedded tissue from each case was microdissected and placed in Eppendorf tubes for gDNA isolation and PCR amplification. Unstained 4 μ m sections were prepared in all cases and utilized for immunohistochemical analysis.

Task 3: Convert the methylation-specific PCR for GST- π from gel analysis to homogeneous fluorescence detection.

Task modified/in progress

Our detection method will be more reliable if it can be made quantitative. We will use a PE 7700 Sequence Detector (available in the AMC Molecular Biology Core) for quantitative PCR of DNA from tissue samples. We will test both Taqman oligonucleotides and Sunrise primers. Taqman probes have the intrinsic advantage of discriminating against primer dimer products, improving the signal to noise ratio, but there are only a limited number of compatible probes possible in the methylation region. There are more options with the Sunrise type of probe. It may be possible, and ultimately cheaper, to use SYBR green detection of product from methylated DNA. Perkin Elmer will be providing software designed to subtract the primer dimer background in such an application, and we are hopeful that this will provide a fall-back if either the Taqman or Sunrise probes are not workable within the constraints of the GST- π methylation region.

In the final form of the diagnostic assay we intend to use multiplex quantitative PCR in which a probe detecting total modified DNA will generate a baseline for quantitation of methylated DNA. The ability to make a direct comparison between total amount of DNA versus methylated DNA in the same sample will improve confidence in both positive and negative judgements.

Task 4: Isolate gDNA from each of the ≥ 220 prostate tissue samples and test each one for GST- π methylation status using the tube based Sunrise™ GST- π methylation assay.

Task in progress

We have begun analyzing tissue samples using the gel-based method as a prelude to development of a tube-based quantitative PCR assay (see Task 3). DNA was extracted from paraffin embedded tissue, using the "x-wax" method followed by bisulfite modification. Using the 466/468 primer pair, we tested 24 samples in an initial trial. To improve detection we re-amplified the first round PCRs. We obtained 7 positives (one not shown) out of 18 samples which had been categorized as malignant by histology. There was one positive among the six samples which had been categorized as benign (Figure 1).

Problems associated with completion of task:

Continued improvement of methylation-specific PCR.

(A) Development of a high sensitivity method for the detection of rare circulating tumor cells will require a very low background signal from the vast excess of normal DNA in a blood sample. Although early results with a methylation-specific PCR detection system were very promising, we were dissatisfied with the level of discrimination between methylated and non-methylated DNA achieved using the primers we started out with, and with the presence of extra non-specific gel bands. Hence we have improved our analysis by testing many alternative mDNA-specific primer pairs. Although primer optimization is assisted by useful computer software (e.g.

"Primer Express" and OLIGO), primer design is still partly empirical. Hence many pairs which would appear to be discriminatory on paper were found to be partly or fully indiscriminate, using as model templates bisulfite-modified DNA from the DU145 and LNCaP.F6C strains as well as CG methylated and non-methylated DNA from the cloned GST- π promoter. From the large group of primers we tested (Figure 2), two pairs have emerged as highly useful and we are in the process of evaluating others. We will have exhausted the list by the beginning of the summer.

(B) A second way of reducing non-specific (i.e. non-methylated) signal is to eliminate the bulk of the non-methylated GST- π template DNA by pre-digesting with methylation-sensitive restriction enzymes which have sites in the region of interest (NotI, SmaI, NgoMIV, FspI). We will develop this simple modification of the method in the near future.

(C) A third method for increasing the "signal-to-noise" ratio is by re-amplification of products, using a second pair of discriminatory primers with sites internal to the first pair. The two pairs of primers we are currently working with (466/468 and 425/429) are not compatible in this sense (they are not "nested"), so a second pair internal to 466/468 is being sought. However we have been able to improve detection by re-amplification with the same primer (see below). We anticipate that nested re-amplification will enhance detection of DNA from rare cancer cells in blood samples. Ultimately we need to be able to detect a few methylated template molecules, and with our current detection we are within an order of magnitude of that.

Discrimination between tumor and benign cells will only be reliable when we can establish confidence limits for positive and negative findings. A negative result is

meaningful only when a satisfactory positive control is included, i.e. showing that the extracted, bisulfite-modified DNA sample is of sufficient quantity and quality to have generated a product if it were methylated. Hence we will use primers specific for modified DNA from the region of interest to assess each sample. These primers need only distinguish efficiently between modified and non-modified DNA and they work very well.

Task 5: Develop an immunohistochemical staining method for GST- π expression.

Task accomplished

In our attempt to develop a novel automated method for immunohistochemical analysis, we used a commercially available rabbit polyclonal antihuman GST- π antibody (Vector Laboratories, Inc., Burlingame, CA). The staining protocol was developed and optimized based on the immunoreactivity of bile duct epithelium in normal human liver tissue (positive control). This unique automated IHC method is an indirect biotin-avidin system. Immunoperoxidase procedures such as this which allow direct light microscopic visualization of antigen in tissue sections are complex and involve precise optimization of various intermediate steps to enable both sensitive and specific detection of the antigen of interest. Additionally, the unique advantage of automating a test procedure is the resultant high levels of reproducibility and validity of the results.

The final IHC protocol we developed and validated utilized the Ventana ES (Ventana Medical Systems, Inc., Tucson, AZ) automated IHC instrument. The tissue sections were deparaffinized, rehydrated and preincubated in 3% hydrogen peroxide. No antigen retrieval step was employed for the detection of GST- π protein. Slides were

positioned in slide holders within the reaction chamber of the instrument. Negative control slides were included to establish background and non-specific staining of the primary and secondary antibodies and/or detection kit reagent. Through the Ventana reagent dispense system, endogenous peroxidase activity was further blocked with 3% hydrogen peroxide. Endogenous biotin was masked by the binding of avidin. Sections were incubated with the primary antibody at a dilution of 1:150 at 42°C for 32 minutes. Subsequent incubations included biotinylated rabbit immunoglobulin secondary antibody, avidin horseradish peroxidase conjugate and diaminobenzylidine (DAB) followed by copper sulfate enhancement and finally counterstaining with hematoxylin. Slides were removed from the instrument, dehydrated, coverslipped and examined for expression of the GST- π protein using a standard light microscope.

Task 6: Test prostate tissue using an immunohistochemical staining method for GST- π expression. Review slides for location of the antibody stain and tissue pathology.

Task accomplished

GST- π protein expression data was generated on the 220 prostate tissues. Immunoreactivity for this protein was scored based on both staining intensity and distribution. The staining intensity was subjectively graded as weak, moderate or intense while the distribution in tumor cells was scored as focal (<10%), regional (11-50%), or diffuse (>50%). The overall results in each case were characterized as either positive or negative for GST- π expression.

In benign epithelium, staining was noted in the cytoplasm of the secretory epithelial cells of prostatic acini in 100% of cases (Figure 3). Additionally, the basal cells of prostatic acini demonstrated an intense, diffuse positivity for this protein (Figure 4) in all cases. The other elements that were immunoreactive for this protein included seminal vesicles (Figure 5), nerves (Figure 6) and ganglia. While the location of staining was predominantly cytoplasmic in the epithelial cells, both nuclear and cytoplasmic staining was noted in the basal cells. In all cases, the basal cell staining was more intense as compared with the epithelial cell staining. Additionally, in no case, was epithelial cell staining observed in the absence of basal cell staining.

In prostatic carcinomas, 97% of cases demonstrated loss of GST- π expression within the tumor cells (Figure 7). In all these cases, there were admixed benign prostatic elements that were positive for this protein, serving as internal controls. The remaining five PACs were positive for GST- π (Figure 8). Foci of high grade prostatic intraepithelial neoplasia (PIN) amongst these tumors also showed absence of immunoreactivity for this protein (Figure 9). Statistical analysis revealed no correlation of loss of GST- π expression with any of the prognostic variables including grade, stage, serum PSA and disease recurrence.

Task 7: Develop an in-situ PCR version of the Sunrise™ GST- π methylation assay.

Task not begun. Rescheduled for year 2. Immediate objective is to find an optimum primer pair for Sunrise application.

Task 8: Test prostate tissue using the in-situ version of the Sunrise™ for GST- π methylation assay. Review slides for location of the fluorescence signal from the in situ PCR assay and tissue pathology.

Task not begun. Rescheduled for year 2.

Task 9: Compare all GST- π methylation and expression data gathered on the \geq 220 prostate tissue samples with the existing information already known about those samples. Write up the results and submit them to a journal such as Cancer Research for peer-reviewed publication.

Task begun, awaiting completion.

Task 10: Develop a DNA isolation/bisulfite modification method for small (pg-ng) quantities of DNA in serum.

Task not begun. Scheduled for year 2.

Task 11: Collect \geq 50 pre-operative serums with known PSA values and post-operative prostate tissue histology.

Task originally scheduled for year 2.

Task 12: Develop an improved sensitivity Sunrise™ GST- π methylation assay for DNA isolated from serum.

Task originally scheduled for year 2.

Task 13: Test a set of ≥ 50 prospective serum samples with known PSA value and prostate tumor status using the high sensitivity version of the Sunrise™ GST- π methylation assay developed in Task 12.

Task originally scheduled for year 2.

Task 14: Compare the GST- π methylation data gathered on the ≥ 50 pre-operative serums with the pre-operative serum PSA value and with prostate tissue pathology results. Write up the results and submit to a journal such as Cancer Research for peer-reviewed publication.

Task originally scheduled for year 2.

Discussion

The results of the current study have yielded several findings that support our hypothesis on the clinical and pathological utility of GST- π in prostate cancer. GST- π protein is expressed in all benign acini and is significantly downregulated in the vast majority of carcinomas. This data supports the findings of others (6, 7) and indicates its promising role as a specific marker of prostate cancer that can be utilized to effectively screen for this disease in the target population. However, given the almost universal loss of GST- π protein in carcinomas, there was no correlation with any of the prognostic variables, indicating that abnormal GST- π expression is independent of tumor biology in prostate cancer. The diffuse intense GST- π immunoreactivity in the basal cells of benign and reactive prostatic acini while being completely absent in both high grade PIN and carcinomas is of particular interest. The fact that basal cells are precursor stem cells to the secretory acinar cells from which carcinomas arise, it becomes clear that the protein downregulation occurs in the early stages of cancer evolution. Since high grade PIN is a forerunner of invasive carcinoma, the ability to detect prostate cancer at this stage by demonstrating abnormal GST- π will significantly impact on early diagnosis and successful management of this disease.

Also of significance is the potential utility of GST- π protein downregulation in diagnosing prostatic carcinoma on small preoperative core biopsies of the prostate. In the last decade, there has been great advancement in our understanding of benign lesions of the prostate that mimic adenocarcinoma. Some of these lesions (Table 1) are not infrequently represented on the 18 gauge needle biopsy tissues causing

confusion with carcinoma. In other situations, extremely tiny amounts of carcinoma may be represented in the biopsy material that may be quantitatively insufficient for a definitive diagnosis on morphologic grounds. In both of these scenarios, the phenomenon of intense GST- π staining of the basal cells and its absence in the neoplastic epithelial cells will aid in differentiating benign from malignant prostatic elements.

With regard to abnormalities of the GST- π gene in prostate cancer, previous studies demonstrated hypermethylation of the CpG sites of the promoter region (1). Using DU145 and LNCaP prostate cancer cell lines, we achieved a desired level of discrimination between non-methylated and methylated GST- π DNA with the final set of primers utilized to date. However, when these primers were applied to PCR amplification of DNA extracted from human prostate cancers, GST- π gene methylation was demonstrated in approximately 40% of cases. Although technical factors cannot be entirely eliminated, this poor detection rate may indicate a heterogeneous pattern of GST- π methylation in human cancers unlike the homogeneous pattern in the above mentioned cell lines. In an effort to increase the sensitivity of this PCR assay, we are currently evaluating the use of multiple primer pairs, nested PCR reamplification and use of methylation sensitive restriction enzymes to eliminate non-methylated DNA. The single methylation positive sample detected among the benign DNA samples may reflect a spordic result or represent a significant fraction of atypical methylation events in normal DNA. Examination of larger numbers of specimens will clarify the situation. If accomplished, demonstrating GST- π abnormalities may significantly aid in screening

and early diagnosis of prostate cancer resulting in enormous savings in health care related expenses.

Key Research Accomplishments

- Assembled 180 prostatic adenocarcinomas and 20 cases each of BPH and normal prostatic tissue with all relevant clinical and pathologic data pertaining to each case from the established data base at the Albany Medical College.
- Microdissected tissue samples from all cases for gDNA isolation for the tube based GST- π methylation assay and cut unstained slides for immunohistochemical staining and subsequent analysis.
- Developed a unique, automated IHC staining protocol for GST- π protein expression, stained all cases and evaluated in detail for the presence and distribution of this protein in both benign and malignant prostatic tissues.
- Developed gel-based PCR detection method for malignant prostatic tissues using optimal primers selected after screening a large set of candidate pairs; sensitivity augmented by PCR re-amplification.
- Analyzed 23 prostate tissues (18 carcinomas, 5 benign) for GST- π methylation using this gel-based PCR assay.

Reportable Outcomes

Manuscripts

Two manuscripts as titled below are currently under preparation for submission for publication in the following journals within the next 6 weeks:

- GST- π protein is downregulated in high grade PIN and prostatic adenocarcinoma and is associated with hypermethylation of the promoter region of the gene (Cancer Research).
- GST- π protein expression correlates with basal cell phenotype in prostatic tissue and is downregulated in high grade PIN and carcinoma (AJSP).

Presentations

- Dr. Ross presented "Prognostic Factors in Prostate Cancer" as Grand Rounds at the Massachusetts General Hospital, Boston in June 1999 highlighting the preliminary data concerning GST- π hypermethylation in prostate cancer.
- Dr. Ross included preliminary immunohistochemical data in the following workshop presentations:
 - American Society of Clinical Pathologists National Meeting in Orlando, FL in April 1999.
 - American Society of Cytopathologists National Meeting in Sacramento, CA in November 1999.

- United States and Canadian Academy of Pathology National Meeting
in New Orleans, LA in March 2000.

Additional Planned Study

1. Comparison of the utility of GST- π and 34 β E12 basal cell immunoreactivity in the diagnosis of prostatic adenocarcinoma and its mimics in needle biopsy specimens..

Conclusions

In summary, we demonstrated that almost all prostatic carcinomas show loss of GST- π protein and established an association with hypermethylation of the promoter region of this gene. Hypermethylation of this site may be relatively heterogeneous in human prostate cancers as compared to prostate cancer cell lines and may require modified PCR assays for enhanced sensitivity. GST- π may constitute a relatively specific marker of this cancer and can be effectively utilized in screening procedures. The diffuse expression of GST- π protein in basal cells but not in neoplastic epithelial cells indicates its early role in the evolution of prostate cancer. The basal cell staining property of GST- π can be of significant help in the accurate and definitive diagnosis of prostatic carcinoma on preoperative needle biopsies.

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APPENDICES

TABLE 1

Mimics of Prostatic Adenocarcinoma

- I. Hyperplastic lesions
 - Atypical adenomatous hyperplasia
 - Clear cell cribriform hyperplasia
 - Sclerosing adenosis
 - Atypical basal cell hyperplasia
- II. Atrophy related lesions
 - Atrophy
 - Post atrophic hyperplasia
- III. Dysplastic lesions
 - High grade PIN
- IV. Normal Structures
 - Cowpers glands
 - Paraganglia
 - Seminal Vesicle

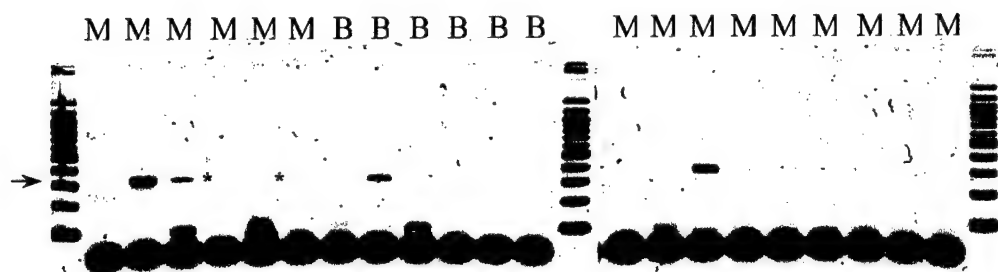


Figure 1: Detection of methylated GST- π promoter DNA in malignant and benign samples. 24 Samples scored for the presence of malignant cells by histology and by immuno-staining were tested for the presence of methylated DNA. DNA was extracted from paraffin- embedded tissue, bisulfite-modified and purified. Samples were amplified in a PCR reaction primed by the 466/468 primer pair and re-amplified with the same primers. Samples from malignant tissue are indicated with an *M*, benign with a *B*. Lanes containing re-amplified products are shown beneath lanes for the first reaction for each sample.

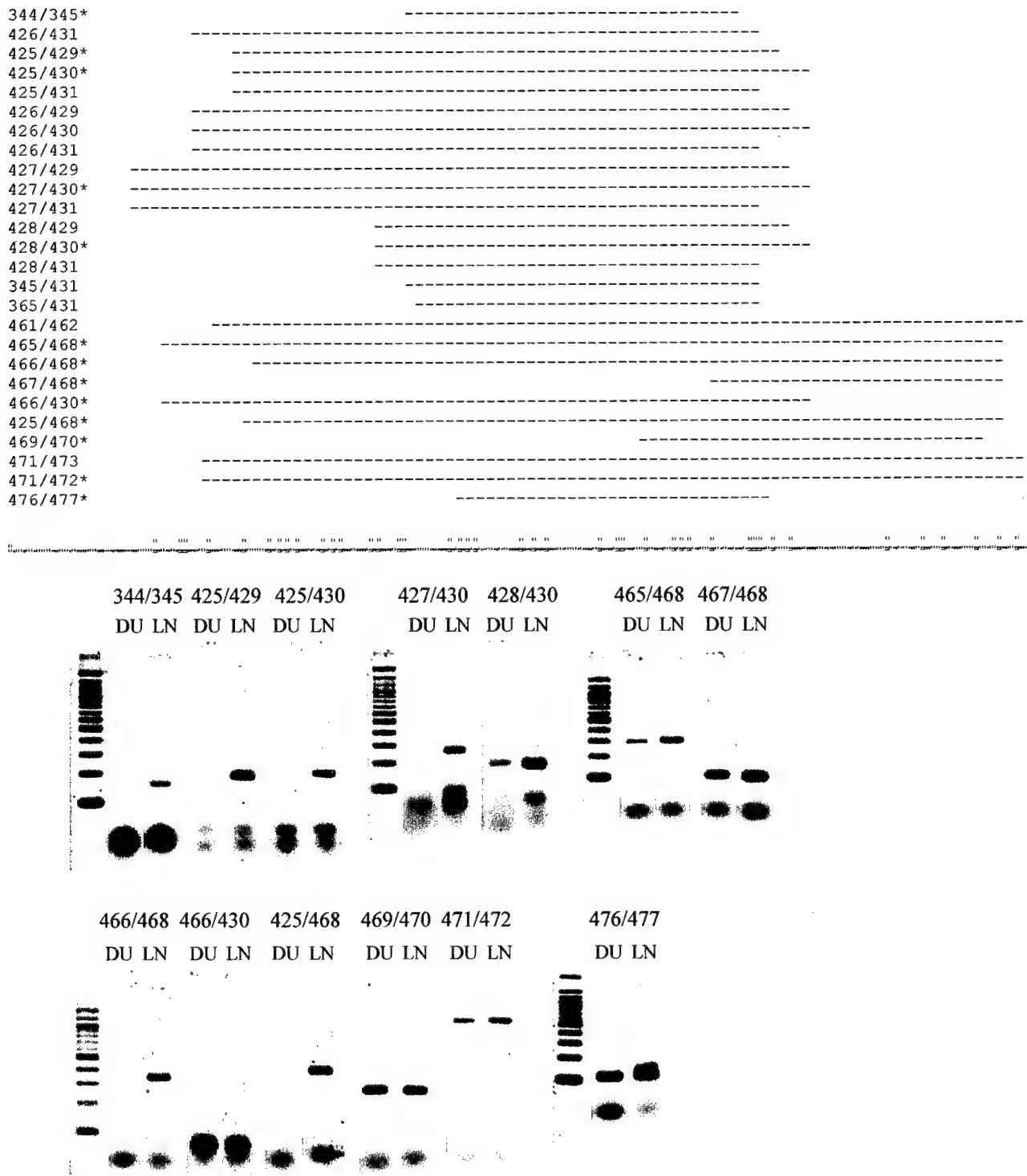


Figure 2: Discrimination (and non-discrimination) of methylated and non-methylated GST- π DNA from tissue culture cells by PCR with various primer pairs.

DNA derived from the DU145 and LNCaP.F6C cell lines (non-methylated and methylated respectively) was bisulfite-modified and used as template for PCR reactions with various primers. The endpoints of the PCR products for each primer pair are shown in the line drawing; the positions of methylatable CG sites are indicated.



Figure 3: GST- π immunoreactivity in benign prostatic acini. Panel A illustrates intense, diffuse, predominantly cytoplasmic positivity in the secretory epithelial cells (100X). Panel B shows scattered nuclear expression of GST- π protein in sporadic acini (400X). (anti-human GST- π , DAB, hematoxylin)

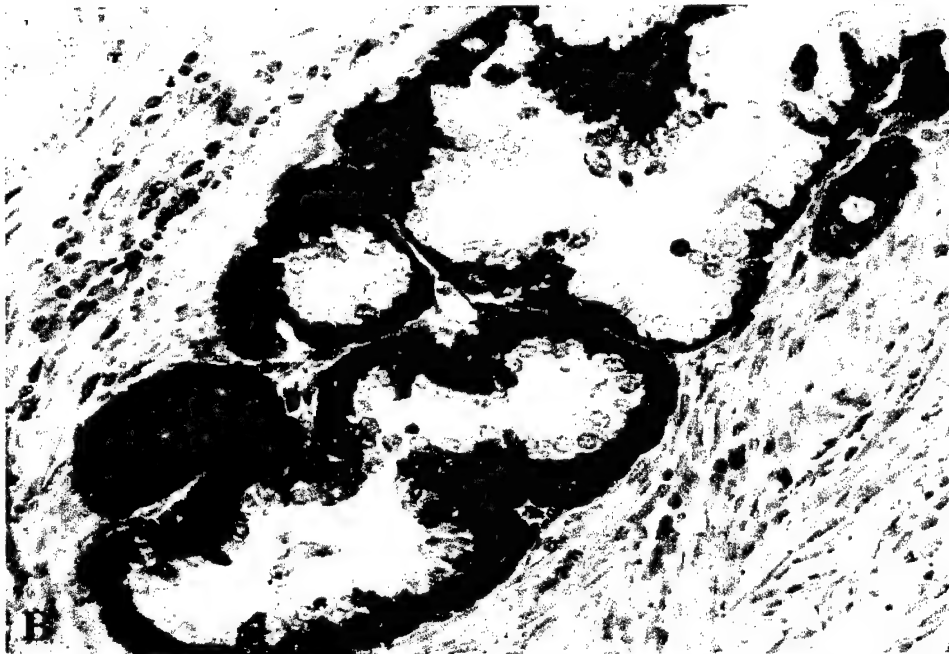
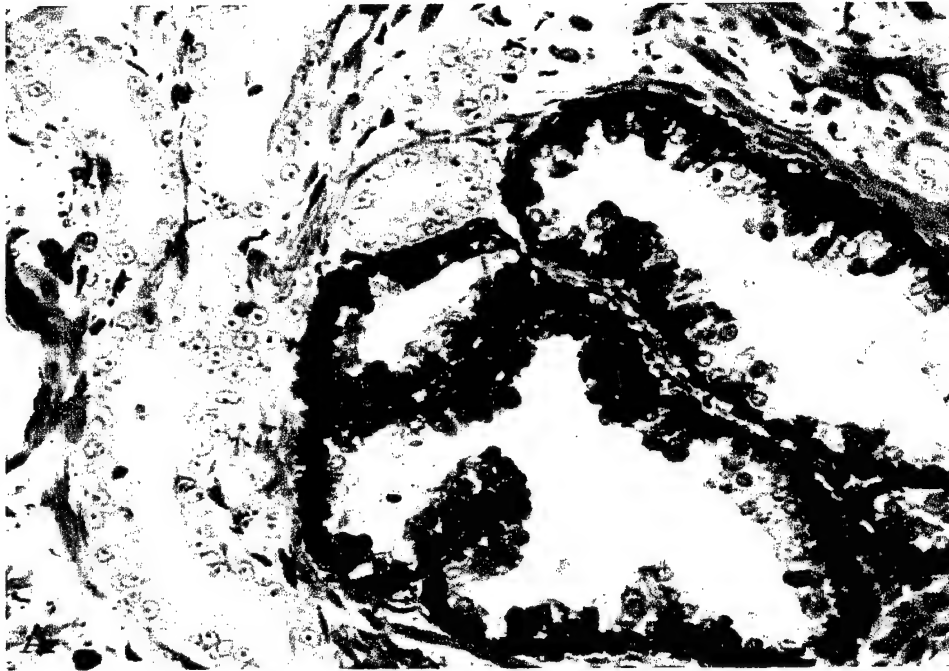


Figure 4: Panels A & B (200X) Intense, predominately basal cell GST- π immunoreactivity in benign prostatic acini. Panel A illustrates focal cytoplasmic and nuclear patterns of positivity in secretory epithelial cells. Also note in Panel A, a focus of low grade prostatic adenocarcinoma (Gleason score 6/10) shows loss of expression of the GST- π protein. (anti-human GST- π , DAB, hematoxylin)

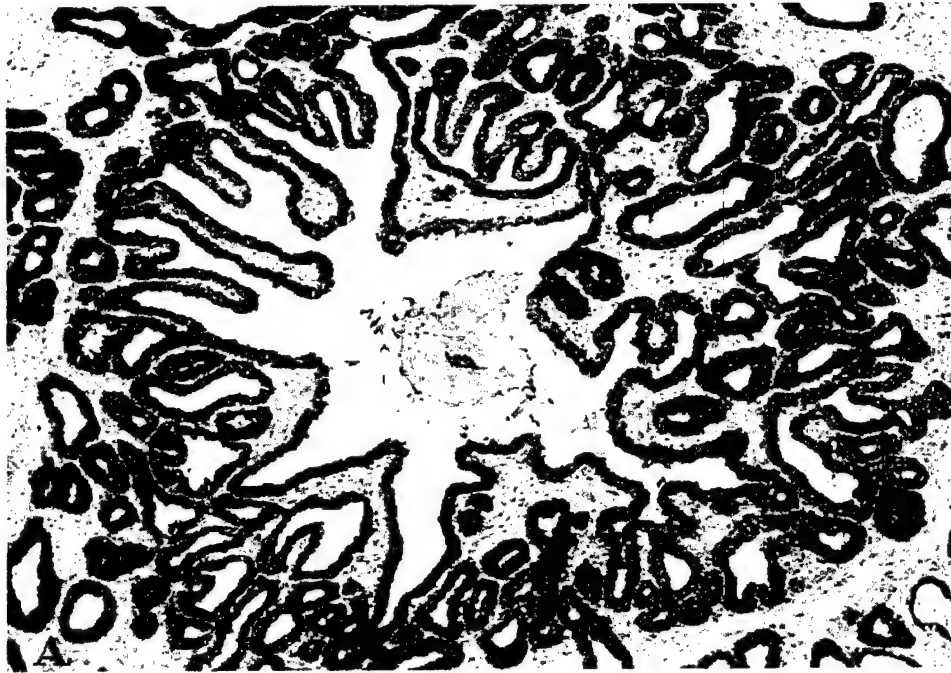


Figure 5: Panel A illustrates a normal seminal vesicle showing intense, diffuse positivity for the GST- π protein (100X). Panel B illustrates complete loss of GST- π protein expression in infiltrating prostatic adenocarcinoma (Gleason grade 6/10) adjacent to a portion of strongly positive seminal vesicle (100X). (anti-human GST- π , DAB, hematoxylin)

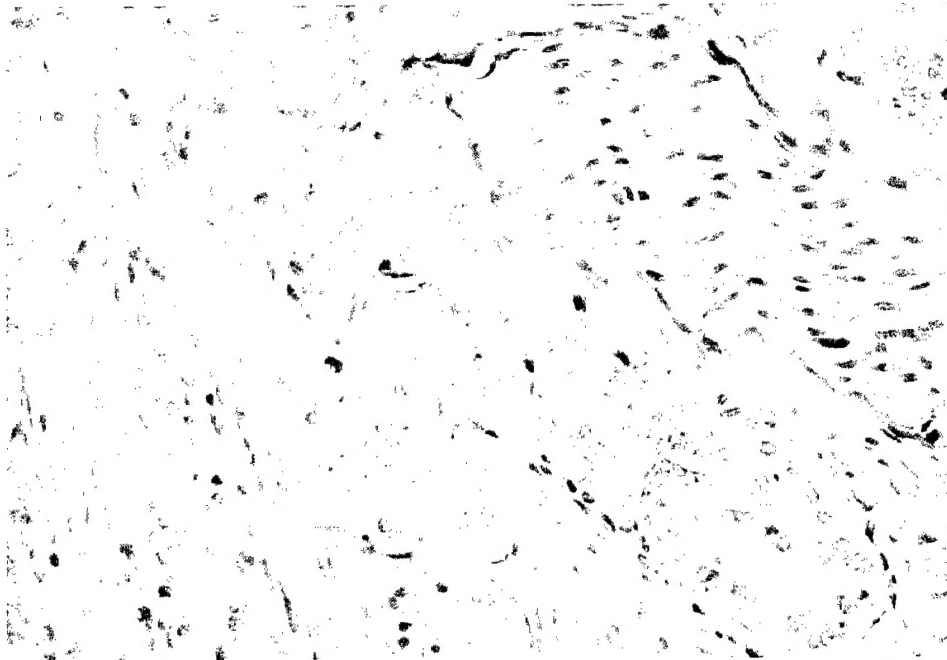


Figure 6: Example of a nerve showing immunoreactivity for the GST- π protein in comparison to the infiltrating prostatic adenocarcinoma which is negative for this protein (200X). (anti-human GST- π , DAB, hematoxylin)

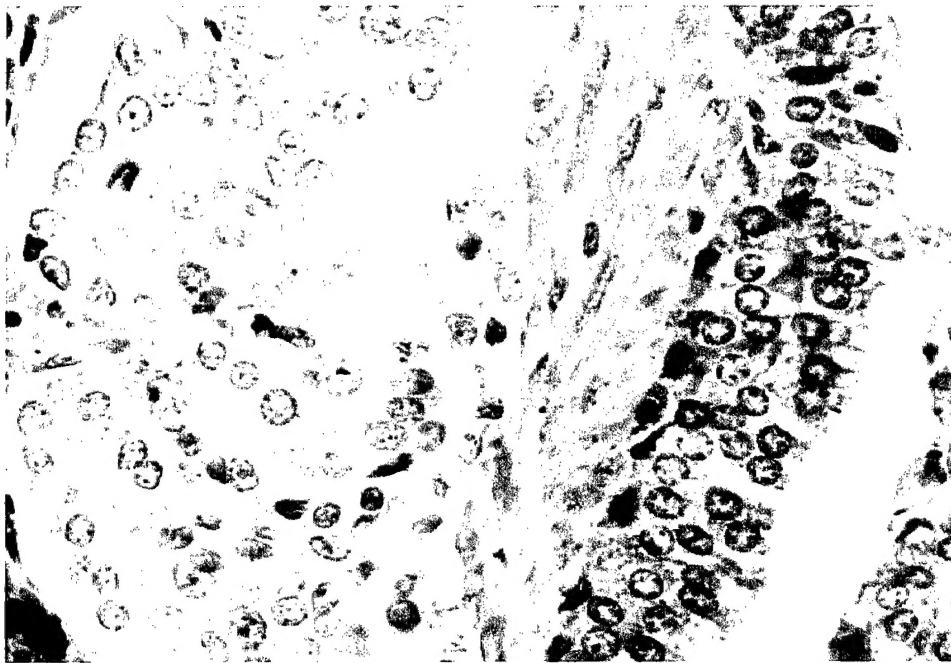
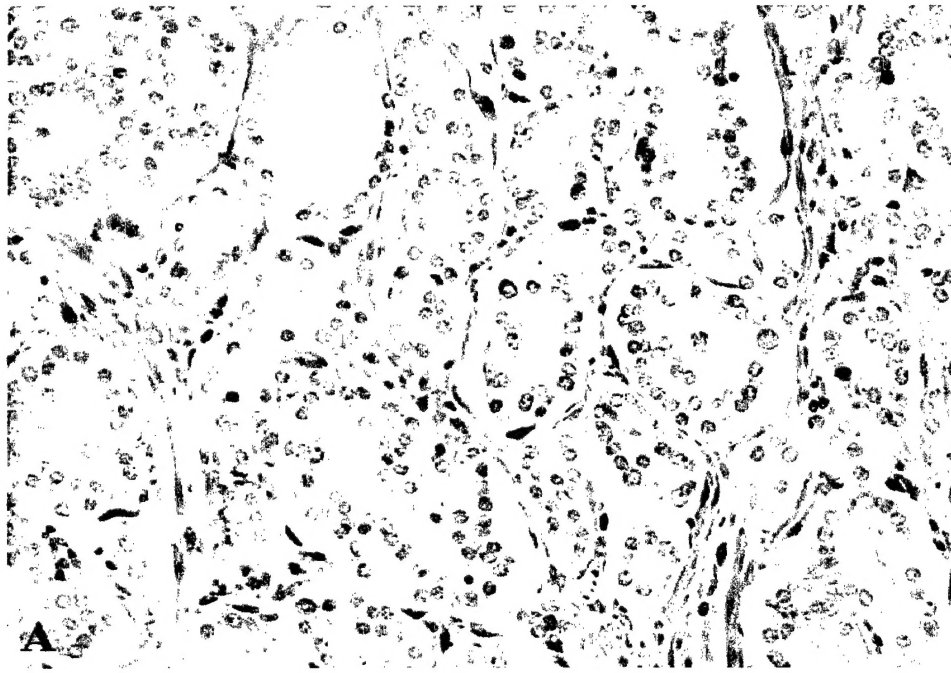


Figure 7: Panels A (200X) & B (400X) An example of low grade prostatic adenocarcinoma showing absence of GST- π protein expression. Note in Panel B, adjacent benign acinus is positive for this protein. (anti-human GST- π , DAB, hematoxylin)

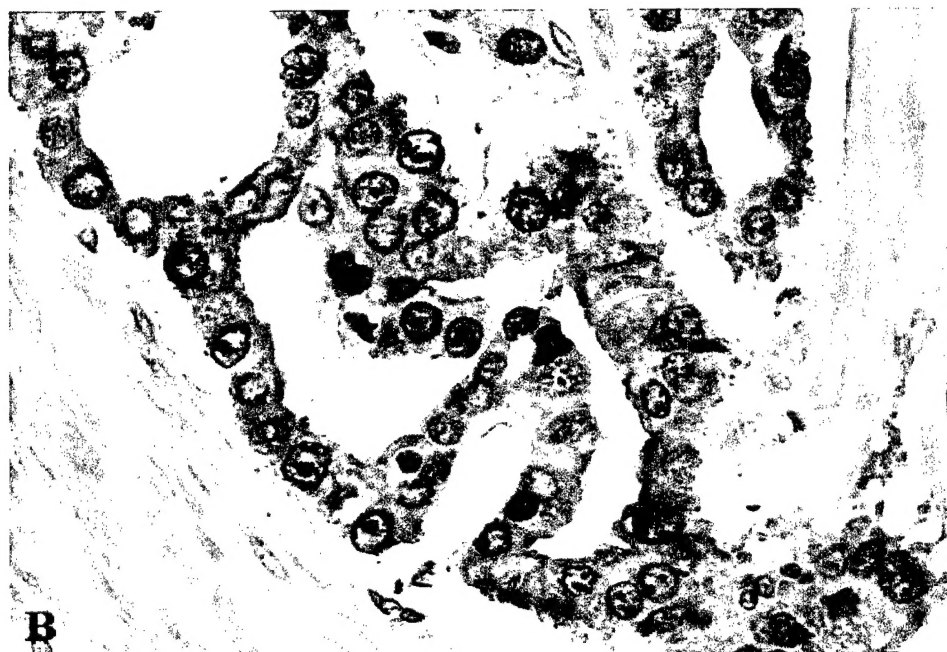
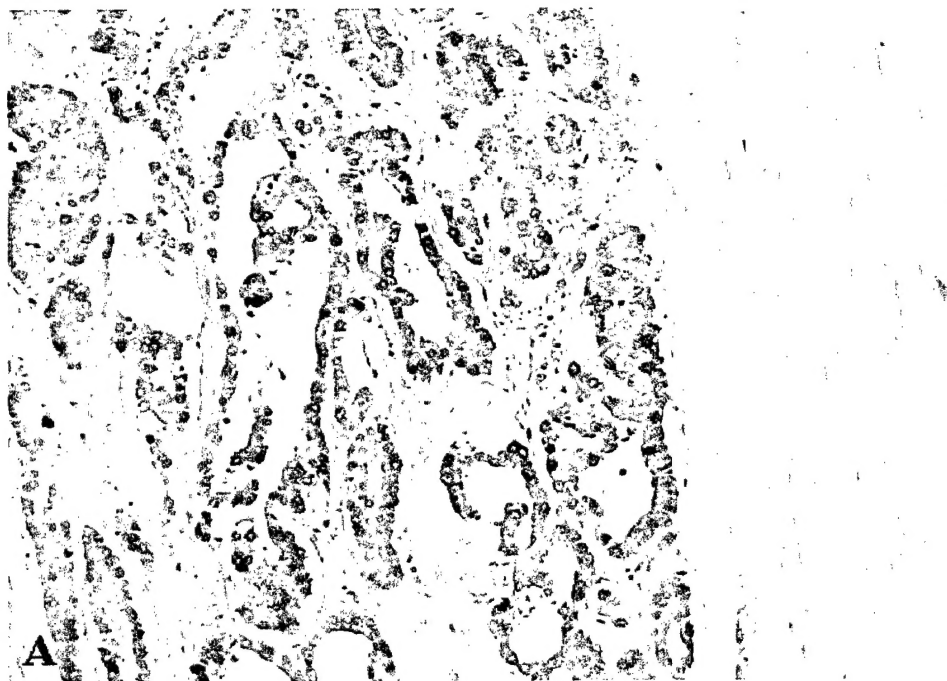


Figure 8: Panels A (200X) & B (400X) A rare example of a prostatic adenocarcinoma showing intense, diffuse, predominately cytoplasmic positivity for the GST- π protein. (anti-human GST- π , DAB, hematoxylin)

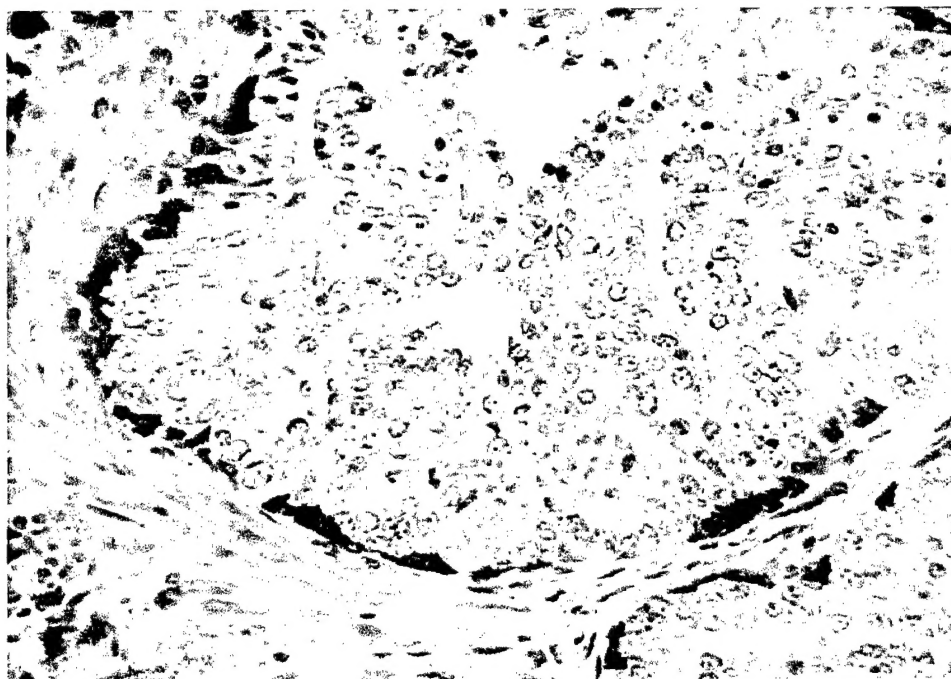


Figure 9: High grade PIN showing absence of expression of the GST- π protein. Note the intense positivity of the basal cells for this protein (200X). (anti-human GST- π , DAB, hematoxylin)